HIV-1-derived single-stranded RNA acts as activator of human neutrophils

Running title: ssRNA40 and activation of human neutrophils.

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Abbreviations:

PRRs: Pattern recognition receptors; TLRs: Toll-like receptors; ROS: Reactive oxygen species; NETs: Neutrophils extracellular traps; ssRNA: single-stranded RNA; HESNs: HIV-1-exposed seronegative individuals; RLRs: RIG-I-like receptors.

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Abstract

Neutrophils are key effector cells of the innate immune system and are involved in the host defense against invading pathogens such as viruses. Recently, it was reported that HIV-1-neutrophil interaction triggers neutrophil activation and promotes expression of Toll-like receptors (TLR). Here, we assessed the role of single-stranded RNA40 (ssRNA40) derived from HIV-1 in neutrophil activation. We observed functional activation of neutrophils in response to HIV-1-derived ssRNA40 based on the expression of TLR7/8, RIG-I and MDA5, induction of cytokines (IL-6 and TNF-α) and the production of reactive oxygen species (ROS). Additionally, ssRNA40 promoted the expression of CD62L and TNF-α and the production of ROS in the presence of the TLR2 agonist Pam_2CSK_4. ssRNA40 together with R848 (an RIG-I/MDA5 agonist) increased CD11b expression but decreased CD62L expression. Furthermore, decreased IL-6 expression was observed in the presence of the TLR4 agonist LPS. Finally, we found that ssRNA40 promotes RIG-I and MDA5 expression in the presence of the TLR2, TLR4 and TLR7/8 agonists. This study demonstrates a functional response of TLRs in neutrophils challenged with ssRNA40, suggesting that TLRs could be involved in the innate immune response observed during HIV infection, which might be mediated by its genome.

KEY WORDS: Neutrophils, TLRs, PAMPs, ssRNA40, cytokines

Introduction

Neutrophils are the first line of defense in response to invading pathogens and trigger innate immune responses. Although their role has been primarily directed towards defense against bacterial infections (1), recent evidence suggests that neutrophils also play a key role during viral infections and exhibit both host defense and pathological functions (2). Therefore, some viral infections can lead to an increase in absolute neutrophil count in the peripheral blood and their migration to the site of infection (3). There is evidence that neutrophil activation by viruses increases the production of chemokines, cytokines (4, 5), and ROS (6, 7). Recently, it was reported that neutrophils activated by HIV-1 exhibited a change in Toll-like receptor (TLR) expression patterns, pro-inflammatory cytokine secretion and ROS production (8). In addition, it was previously demonstrated that neutrophils from HIV-1-exposed seronegative individuals (HESNs) present reduced expression of several pattern recognition receptors (PRRs) and pro-inflammatory cytokines compared to neutrophils from HIV-1 patients (9). Additionally, it was found that neutrophils from HESNs are less sensitive to TLR4 stimulation and produce significantly lower levels of ROS in response to HIV-1. However, the mechanisms underlying recognition or neutrophil activation by HIV-1 or HIV-1 components are unknown. On activated neutrophils, TLRs induce pro-inflammatory cytokine production, CD62L (L-selectin) and CD11b/CD18 expression (10-12) and regulate the chemotactic response (13). Other reports showed an induction of ROS production in neutrophils activated through TLR stimulation (10, 14).

Additionally, NK cells recognize HIV-1 ssRNA40 via TLR7 and TLR8 (15, 16). HIV-1-derived ssRNA40 contributes to the immune activation of NK cells and plays an important role in HIV-1 infection (16). Since neutrophil activation in response to HIV-1 stimulation has been reported, we evaluated here the potential role of ssRNA40 in mediating the activation of neutrophils both alone and in the presence of PRR agonists. We found that expression of neutrophils CD62L and CD11b, ROS production and IL-6/TNF-α secretion were induced by ssRNA40. Furthermore, neutrophils were significantly activated *in vitro* by established TLRs and RIG-I-like receptor (RLRs) agonists in combination with ssRNA40. Finally, we observed increased expression of RLRs in response to specific TLR agonists and ssRNA40. Taken together, our data provide insights into the possible mechanism by which HIV-1 activates neutrophils and the roles that neutrophils play during HIV-1 infection.

Materials and methods

Ethics statement

The study was designed and performed according to the Declaration of Helsinki and approved by the Ethics Committee (Universidad de Antioquia).

Study subjects

Healthy individuals voluntarily agreed to participate in this study and signed a written informed consent form prior to participation. The exclusion criteria included any clinical presentation associated with infectious disease and pregnancy. The inclusion criteria included individuals between 18 and 50 years of age that presented a normal leukocyte count (4,500-11,000 cells/mm^3). The

donors declared that they were nonsmokers and were not taking any medication.

Sampling and neutrophil purification

For neutrophil purification, we followed the strategy previously reported (8). Briefly, blood samples were collected by venipuncture in tubes containing anticoagulant (citric acid, citrate, and dextrose; BD Pharmingen, San Diego, CA, USA). Neutrophils were purified by dextran sedimentation and centrifuged in a Ficoll Hypaque gradient (Sigma-Aldrich, St. Louis, MO, USA). Purified neutrophils were resuspended in RPMI 1640 medium enriched with 10% FBS (fetal bovine serum). The purity of the neutrophil preparation was greater than 94% and the viability was greater than 90% after up to 10 h of culture.

Monoclonal antibodies

Monoclonal antibodies to CD11b conjugated with PE-Cy5, CD62L conjugated with FITC and TLR2 (clone TL2.1) and TLR4 (clone HTA125) conjugated to phycoerythrin were described previously (8). The FcR blocking reagent was obtained from Miltenyi Biotec (Auburn, CA, USA). Conjugated isotype control antibodies were used as controls.

Neutrophil stimulation *in vitro* with singled-stranded RNA40 (ssRNA40) derived from HIV-1

Immediately after purification, $2.5x10^5$ neutrophils/ml were plated onto 96-well plates in RPMI 1640 medium supplemented with 10% FBS and incubated at 37ºC and 5% CO_2. Neutrophils were stimulated with 3 ng/µl of ssRNA40

(Invivogen, San Diego, CA, USA) for 3 h to evaluate mRNA expression and for 8 h to evaluate protein expression; A-to-U-replaced ssRNA-41 (a derivative of ssRNA40) was used as ~~the~~ negative control at a concentration of 3 ng/µl (Invivogen, San Diego, CA, USA) (17). Alternatively, to assess the effect of ssRNA40 on PRR expression, neutrophils were challenged with ssRNA40 (3 ng/µl) simultaneously with Pam_2CSK_4 (20 ng/ml), LPS (0.1 ng/ml) or R848 (1 µg/ml), that are agonists of TLR2, TLR4 and TLR7/8, respectively (Invivogen, San Diego, CA, USA). To evaluate RLR expression, neutrophils were stimulated with TLR agonists in the presence of ssRNA40. After stimulation, the neutrophils were harvested and assayed for TLR/RLR mRNA expression by real-time PCR.

Flow cytometry analysis

Flow cytometry was used to evaluate the effects of ssRNA40 on the expression of TLR2, TLR4, CD62L and CD11b on neutrophils as previously described (8, 18). Briefly, freshly isolated neutrophils were surface-stained with the appropriate antibodies for 25 min; acquisition was performed immediately using a FACSCan™ flow cytometer (BD Biosciences, San Jose, CA, USA). The acquired events were analyzed using FACS Diva software version 6.1.2. Receptor expression was expressed as the mean fluorescent intensity (MFI) of the overall cell sub-population after subtraction of the isotype control.

RNA isolation, cDNA synthesis and quantitative real-time PCR

The mRNA quantification for TLR2, TLR4, TLR7, TLR8, RIG-I and MDA5 was performed in neutrophils by real-time PCR as previously described (8, 19, 20).

For total RNA preparation, the RNeasy isolation kit was used (Qiagen, Valencia, CA, USA). cDNA was synthesized using the RevertAid Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. The primers used were previously reported (8). The relative expression of each target gene was normalized to the unstimulated control and to the housekeeping gene GAPDH ($^{\Delta\Delta}$Ct) and reported as the fold change.

ELISA

The neutrophil culture supernatants were tested for the production of TNF-α and IL-6 using ELISA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Quantification of reactive oxygen species

ROS production was quantified using dihydrorhodamine 123 (Invitrogen, San Diego, CA, USA) according to the manufacturer's directions and as described previously (8). Briefly, commercial dihydrorhodamine was diluted 1:10 in 1X PBS. Then, the neutrophils were incubated with 10 µl of this dilution in each well (96-well plate) for 8 h at 37°C. After incubation, the cells were harvested, washed with PBS, centrifuged for 5 min at 1,800 rpm, and resuspended in PBS. Acquisition was performed immediately using a FACSCan™ flow cytometer (BD Biosciences, Pharmingen, San Jose, CA, USA). Analyses were performed using FACSDiva software (BD Biosciences, Pharmingen, San Jose, CA, USA).

Statistical analyses

All data were plotted and analyzed using GraphPad Prism 5.0 (GraphPad Software Inc. San Diego, CA, USA). Comparisons were performed using the Wilcoxon test between the data of interest as specified in each figure legend. The data are represented as median and range. Significant results are defined as $p<0.05$ (*), $p<0.01$ (**) and highly significant results as $p<0.001$ (***).

Results

ssRNA40 decreases TLR2 mRNA expression but increases TLR7/8, RIG-I and MDA5 mRNA expression in neutrophils

To determine whether HIV-1 RNA plays a role in increasing TLR mRNA expression in human neutrophils, we evaluated the effects of short sequences of single-stranded GU-rich RNA derived from HIV-1 (ssRNA40) on the mRNA expression of TLR2, TLR4, TLR7 and TLR8 and the RLRs RIG-I and MDA5. As control, the neutrophils were triggered only with the respective TLR agonists or the ssRNA41 control.

The results showed a significant decrease in TLR2 mRNA upon stimulation with ssRNA40 compared with the ssRNA41 control (Fig. 1a); however, no effect was observed on TLR4 mRNA expression (Fig. 1b). In contrast, an increase in TLR7 and TLR8 mRNA expression was observed when the neutrophils were treated with ssRNA40 compared with the control (Fig. 1c and 1d) as previously reported (17). The TLR2 and TLR4 protein expression levels were assessed but no changes were observed upon stimulation with ssRNA40 (Online resource 1a and b). Additionally, extracellularly delivered ssRNA40 significantly increased

the mRNA expression of the other PRRs involved in the innate immune response to viral agents, such as RIG-I and MDA-5, compared with the ssRNA41 control (Fig. 1e and f). To the best of our knowledge, this is the first report to show increased mRNA expression of these two RLRs in response to ssRNA40 treatment.

Activation of neutrophils with ssRNA40 results in enhanced pro-inflammatory cytokine secretion and ROS production

To determine whether treatment with ssRNA40 promoted neutrophil activation, these cells were stimulated with ssRNA40, and CD62L and CD11b were evaluated by flow cytometry after 8 h of treatment. We found that ssRNA40 treatment significantly decreased CD62L expression (Fig. 2a). No significant changes were observed for the expression of CD11b, but an increasing trend was detected. Next, we examined whether ssRNA40 induced pro-inflammatory cytokine secretion from neutrophils. Fig. 2c and 2d show that the ssRNA40 treatment induced a significant increase in both IL-6 and TNF-α expression compared with ssRNA41 stimulation. Finally, we investigated whether ssRNA40 induced ROS production and observed a significant increase compared with the control (Fig. 2e). Similar results were observed when the neutrophils were stimulated with HIV-1 RNA purified from H9 HTLVIII cells (*i.e.,* HIV-1 RNA); i.e. up-regulated IL-6 (Online resource 2a) and TNF-α (data not shown) secretion and significantly increased ROS production (Online resource. 2b).

Co-stimulation of neutrophils with ssRNA40 and TLR agonists leads to TLR and RLR mRNA expression

Because a crossed effect between TLRs was previously reported (8, 21), neutrophils were stimulated only with their respective TLR agonist or simultaneously with ssRNA40 to evaluate the effect of ssRNA40 on TLR mRNA expression upon stimulation with specific agonists. Co-stimulation with ssRNA40 and the TLR2, TLR4 and TLR7/8 agonists induced significant up-regulation of the TLR2 and TLR4 mRNAs versus stimulation with the TLR agonists alone (Fig. 3a and 3b). Subsequently, we investigated whether TLR2 and TLR4 mRNA expression resulted in the expression of the TLR2 and TLR4 proteins in neutrophils. Flow cytometry analyses demonstrated a significant increase in TLR4 protein expression in neutrophils co-stimulated with Pam_2CSK_4 and ssRNA40 (Online resource. 3b). Following evaluation of the different co-stimuli (ssRNA40 and TLR agonists), we found that neutrophils stimulated with Pam_2CSK_4 and ssRNA40 exhibited significantly increased TLR7 and TLR8 mRNA levels (Fig. 3c and 3d). Based on our results on RIG-I and MDA5 mRNA expression in response to ssRNA40 (Fig. 1e and 1f), and on a previous report (22), we extended our study to evaluate the effects of the TLR agonists on the expression of these PRRs. Therefore, neutrophils were co-stimulated with a TLR2, TLR4 or TLR7/TLR8-specific agonist, and ssRNA40 and the RIG-I and MDA5 levels were determined. ssRNA40 significantly up-regulated RIG-I mRNA expression in the presence of Pam_2CSK_4, and R848 but not of LPS (Fig. 3e). Moreover, co-stimulation of neutrophils with Pam_2CSK_4 or

co-stimulation of LPS with ssRNA40 significantly increased MDA-5 mRNA expression levels (Fig. 3f).

Co-stimulation with TLR agonists and ssRNA40 results in neutrophil activation, cytokine secretion and ROS production

We then investigated whether the effect observed in modulation of TLRs and RLRs in neutrophil co-stimulation were reflected in neutrophil activation. Interestingly, neutrophils expressed the lowest levels of CD62L following stimulation simultaneously with ssRNA40 and with Pam_2CSK_4 or R848 versus stimulation with the respective TLR agonist alone (Fig. 4a). When CD11b expression was analyzed, a significant increase was observed as a result of simultaneous co-stimulation with ssRNA40 and R848 compared to stimulation with the TLR7/8 agonist alone (Fig. 4b).

Pro-inflammatory cytokine secretion into neutrophil culture supernatants was determined by ELISA. As shown in Fig. 4c, ssRNA40 resulted in significant down-regulation of IL-6 production in the presence of LPS, but no effect was observed with Pam_2CSK_4 or R848 (Fig. 4c). Conversely, there was a significant up-regulation of TNF-α in neutrophils stimulated with ssRNA40 and Pam_2CSK_4 compared to stimulation with the TLR agonist alone (Fig. 4d). Finally, ROS production was significantly induced in neutrophils stimulated simultaneously with ssRNA40 and with Pam_2CSK_4 compared to stimulation with the TLR2 agonist alone (Fig. 4e).

Discussion

There have been reports that unstimulated neutrophils from HIV-infected patients exhibit high expression of the adhesion molecule CD11b, and low expression of CD62L, when compared with those from healthy donors (23). Similarly, spontaneous ROS production was also described in circulating neutrophils (23). Although, the role of HIV-1 RNA in neutrophil activation, pro-inflammatory cytokine secretion and ROS production through signaling of the TLR pathway has not been reported, recently it was shown that the HIV-1-neutrophil interaction triggers neutrophil activation and TLR expression, altering the functions of these cells (8). Here, we demonstrate that neutrophils from healthy donors produced significant amounts of TLR7/8, but decreased TLR2 mRNA expression in response to ssRNA40 derived from HIV-1 RNA. This result for TLR7 and TLR8 was expected because ssRNA was described as its natural ligand (17) and ssRNA40 was previously reported to modulate NK cell activation via TLR7 and TLR8 (16, 17). This result also becomes more important since HIV-1 has been reported to increase TLR4 and TLR7 expression in human neutrophils (8). Moreover, a previous study performed on patients with chronic HIV-1 infection reported that increased mRNA expression of TLR6, TLR7 and TLR8 correlated with the HIV RNA load in the plasma (24), and our results show that ssRNA40 significantly down-regulates TLR2 mRNA expression. Down-regulated TLR2 expression was observed in myeloid dendritic cells obtained from healthy donors and stimulated with HIV-1 (20). Here, we found that stimulation of neutrophils with ssRNA40 resulted in decreased TLR2 expression. This finding could indicate differential regulation of

TLR and RLR expression mediated by ssRNA40, possibly as a mechanism to evade the innate immune response through this receptor. On the other hand, an increase of RIG-I and MDA-5 mRNA expression in response to ssRNA40 derived from HIV-1 RNA was observed. This is the first study to describe this regulation in neutrophils, although previously HIV-1 infection has been reported to result in a significant increase in RIG-I levels in macrophages (25). Induction of RIG-I expression was also shown in PBMCs from HIV-1 patients (26). Furthermore, RIG-I activation induced the expression of intracellular HIV-1 restriction factors, including ISGs, IFN-α/β and IRFs, and inhibited HIV-1 replication in macrophages (27). These and our results reported here highlight the possible importance of RIG-1/MDA5 signaling in anti-HIV innate immunity.

Activation in neutrophils was evaluated based on changes in the expression of adhesion molecules such as CD62L and CD11b that has been extensively described as activation markers of neutrophils. CD62L is expressed at high levels in neutrophils and is involved in neutrophil rolling on endothelium; when activated, CD62L is rapidly shed giving rise to ~~a~~ stronger adhesion mediated by integrins such as CD11b allowing ~~the~~ transmigration (28). Both, CD62L loss and CD11b up-regulation are modulated by TLR stimulation, leading to functional activation, and initiating the inflammatory response (10, 12, 13, 29). We observed that HIV-1 RNA regulated the activation of neutrophils in addition to regulating TLR and RLR expression based on changes in the expression of adhesion molecules, such as the down-regulation of CD62L (Fig. 2a) and the increase in CD11b. This finding is consistent with previous reports showing that HIV-1 ssRNA induced dendritic cell maturation and NK cell activation (16, 30).

Another study observed that neutrophils from HIV-1-infected patients exhibited decreased CD62L expression and increased CD11b expression during the acute phase of infection (high replication rate) (23, 31, 32). It is well known that HIV-1 pathogenesis is characterized by an altered production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α, mediated mainly by mononuclear peripheral blood cells (33, 34). Accordingly, we observed an increased production of IL-6 and TNF-α by neutrophils stimulated with ssRNA40 (Fig. 2c and 2d). This suggests that HIV-1 RNA acts as activator of human neutrophils and supports the hypothesis that neutrophils may actively contribute to cytokine dysfunction in HIV infection.

In addition to cytokine production, ssRNA40 also induced a significant increase in ROS production (Fig. 2e). This phenomenon is one of the main neutrophil mechanisms involved in pathogen control (35). However, little is known about the effects of ROS on viral infections. Some viruses, including influenza virus (36, 37), hepatitis C virus (38), and HIV-1, induce ROS production. In addition, an association was reported between HIV-1 Nef and p22-phox, which is a component of the NADPH oxidase (23, 39-41). Based on our results we propose that neutrophil activation and ROS production may be modulated through the activation of TLRs and RIG-I/MDA5 by HIV-1 RNA. Indeed, a relationship between TLRs and ROS production has previously been demonstrated for HIV-1 (29, 42, 43). Our hypothesis is supported by the observed neutrophil activation and functional responses under simultaneous stimulation with TLR agonists and ssRNA40. Indeed, we showed that co-stimulation with Pam_2CSK_4 or R848 and ssRNA40 significantly amplified

neutrophil activation based on the significant decrease in CD62L and significant increase in CD11b. Because TNF-α secretion and ROS production were upregulated in response to the TLR2 agonist and ssRNA40 but not in the presence of R848 (Fig. 4a, 4d and 4e), we suggest that signaling of TLR2 might be involved in ROS production. Accordingly, neutrophils selective for TLR ligands were reported in bovines, and the authors showed that a TLR2 agonist but not LPS induced ROS production (44). Moreover, ROS production could be involved in HIV-1 pathogenesis because neutrophils from HIV-1-exposed seronegative individuals challenged with HIV-1 and stimulated with the TLR2 agonist produced lower levels of ROS versus neutrophils from HIV-1 patients (9).

Cross-regulation was recently reported to exist between PRRs, leading to either a synergistic, additive or antagonistic response (21, 45). Additionally, cross-regulation between TLRs on neutrophils stimulated with HIV-1 and different TLR agonists was previously reported (8). Consequently, interactions between various PRRs are suggested to be required to ensure an effective innate immune response (46). Here, when addressing potential TLR–TLR and TLR-RLR cross talk, we found that TLR2, TLR4 and TLR7/8 agonists in combination with ssRNA40 had synergistic effects on TLR2 and TLR4 (Fig. 3a-b). For TLR7/8, a synergistic effect occurred only with TLR2 combined with ssRNA40 (Fig. 3c-d). Interestingly, TLR2 and TLR7/8 agonists in combination with ssRNA40 synergistically up-regulated RIG-I mRNA expression, whereas TLR2 and TLR4 agonists combined with ssRNA40 synergistically increased MDA5 mRNA expression (Fig. 3e-f). In concordance with a previous report (47), our

results suggest that cross-regulation occurs not only between TLRs but also between TLRs and RLRs and that this mechanism may help amplify the immune response to HIV-1 infection.

In conclusion, we showed for the first time that TLR and RLR expression was altered in neutrophils in response to ssRNA40 derived from HIV-1, which might result in neutrophil activation, pro-inflammatory cytokine secretion and ROS production; furthermore, we observed a TLRs and RLRs cross-regulation. This behavior may contribute to immune evasion or immune-pathogenic events. However, there are many exciting avenue of research that remain to be explored to fully understand the true role of neutrophils during HIV-1 infection; for example, to determine if the RIG-I and MDA-5 downstream pathways or if the TLR7/8 up-regulation leads to type I interferon production in neutrophils, in response to viral genome.

Competing interests

None of the authors has any potential financial conflict of interest related to this manuscript.

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Figure legends

Fig. 1 ssRNA40 downregulates the expression of TLR2 and increases the expression of TLR7/8, RIG-I and MDA5 in neutrophils.

Neutrophils ($2x10^5$) purified from healthy donors (n=8) were stimulated *in vitro* with 3 μg/ml of ssRNA40 or ssRNA41. The specific agonists for each TLR were used as positive controls (n=6) as follows: Pam_2CSK_4 (20 ng/μl) for TLR2, LPS (0.1 ng/μl) for TLR4 or R848 (1 μg/ml) for TLR7 and TLR8. Stimulations were performed for 3 h prior to the determination of TLR2 (a), TLR4 (b), or TLR7 (c) and TLR8 (d) mRNA expression by real time RT-PCR. Additionally, the mRNA expression of RIG-I (e) and MDA-5 (f) by real time RT-PCR was evaluated. The median and range of each transcript's relative expression versus that of a

housekeeping gene are shown. The data are represented as the mean fluorescence intensity (MFI) of each specific TLR. Comparisons were performed using the Wilcoxon test. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

Fig. 2 ssRNA40 regulates the activation and function of neutrophils. Neutrophils ($2x10^5$) purified from healthy donors (n=8) were stimulated *in vitro* with 3 µg/ml of ssRNA40 and 3 µg/ml of ssRNA41, was used as control. After 8 h of stimulation, the expression of CD62L (a) and CD11b (b) was assessed by flow cytometry, the production of IL-6 (c) and TNF-α (d) was determined by ELISA, and the production of ROS (e) was assessed by flow cytometry (presented as the percentage of neutrophils producing ROS). Comparisons were performed using the Wilcoxon test. Median and range are shown. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

Fig. 3 Co-stimulation of neutrophils with ssRNA40 and TLR agonists enhances TLR and RLR mRNA expression.

A total of $2.5x10^5$ neutrophils obtained from healthy donors (n=8) were co-stimulated with different agonists (20 ng/µl of Pam2CSK4 for TLR2; 0.1 ng/µl of LPS for TLR4; 1 µg/µl of R848 for TLR7/TLR8) and simultaneously with 3 µg/ml of ssRNA40 or 3 µg/ml of ssRNA4. Expression of TLR2 (a), TLR4 (b), TLR7 (c), TLR8 (d), RIG-I (e) and MDA-5 (f) mRNA were evaluated 3 h post-stimulation by real time RT-PCR. The median and range of each transcript versus the

transcript of a housekeeping gene are shown. Comparisons were performed using the Wilcoxon test. Data is represented as median and range. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

Fig. 4 Co-stimulation with TLR agonists and ssRNA40 of neutrophils modulates their activation and functionality.

Neutrophils ($2.5x10^5$) purified from healthy donors (n=8) were stimulated with TLR agonists (20 ng/µl Pam2CSK4; 0.1 ng/µl LPS or 1 µg/µl R848) and were simultaneously stimulated with 3 µg/ml of ssRNA40 for 8 h. The expression of the adhesion molecules CD62L (a) and CD11b (b) was measured by flow cytometry and reported as the MFI. Production of IL-6 (c) and TNF-α (d) was assessed by ELISA, and ROS (e) production by flow cytometry (presented as the percentage of neutrophils producing ROS). Comparisons were performed using the Wilcoxon test. The median and range are shown. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

Online resource 1 Protein expression of TLR2 and TLR4 in neutrophils stimulated *in vitro* with ssRNA40

Neutrophils ($2x10^5$) purified from healthy donors (n=6) were stimulated *in vitro* with 3 µg/ml of ssRNA40 or 3 µg/ml of control ssRNA41. The specific agonists for each TLR were used as positive controls as follows: Pam_2CSK_4 (20 ng/µl) for TLR2, and LPS (0.1 ng/µl) for TLR4. Stimulations were performed for 8 h prior to determining TLR2 (a) and TLR4 (b) expression by flow cytometry. The data is presented as the MFI of each specific TLR. Comparisons were

performed using the Wilcoxon test. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

Online resource 2 Functional activation of neutrophils by viral RNA.

Neutrophils ($2.5x10^5$) purified from healthy donors (n=5) were exposed to viral RNA obtained and purified from viral particles present in H9 HTLVIII chronically infected cell supernatants using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). As a negative control H9 cell supernatant was used; it was processed and treated the same way as the supernatant H9 HTLVIII cells. IL-6 secretion was quantified by ELISA (a). In addition, ROS production (b) was measured by flow cytometry and is reported as the percentage of neutrophils producing ROS. Comparisons were performed using the Wilcoxon test. The median and range are shown. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

Online resource 3 Protein expression of TLR2 and TLR4 in neutrophils co-stimulated with TLR agonists and ssRNA40

A total of $2.5x10^5$ neutrophils were stimulated simultaneously with TLR agonists (20 ng/µl Pam2CSK4, 0.1 ng/µl LPS or 1 µg/µl R848) and ssRNA40 (3 µg/ml) for 8 h. Protein expression of TLR2 (a) and TLR4 (b) was determined by flow cytometry. The data are presented as the MFI of each specific TLR. Comparisons were performed using the Wilcoxon test. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).